A native picrotoxin-resistant GABA-gated chloride channel receptor subtype in cockroach neurons†

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Abstract: Among insect GABA receptors, the GABA-gated chloride channel subtype is insensitive to bicuculline and has been thought to be composed of two populations because of differences in chloride conductance increase, GABA and picrotoxin (PTX) sensitivity. To characterize this possible diversity in GABA-gated chloride channels, electropharmacological experiments were performed on giant interneuron synaptic GABA receptors and on somatic GABA receptors of dorsal unpaired median (DUM) neuron and fast coxal depressor (D_f) motoneuron of the cockroach *Periplaneta americana* (L). Electrophysiological assays performed at cercal-afferent giant interneuron synapses demonstrated that a biphasic increase in membrane conductance, in response to long-lasting (30 s) neuropilar microapplication of GABA, could be explained by the existence of two GABA-operated chloride channel receptor subtypes. The low stable membrane conductance increase, representing less than 30% of the maximum reached during the early transient phase, was not desensitized quickly. It was reproduced by neuropilar microapplication of *cis*-4-aminocrotonic acid (CACA) and, in contrast to the fast phase, was not antagonized by bath application of 10^{-5} M PTX. Long-lasting (3 min) pneumatic pressure application of GABA on the cell body of motoneuron D_f evoked a fast transient hyperpolarization followed by a slower phase of further hyperpolarization. PTX (10^{-5} M) blocked the fast transient phase and revealed a slow stable hyperpolarization. PTX (10^{-4} M) blocked the major part of the remaining GABA response. The slow hyperpolarization was reproduced by application of CACA. Similar effects of GABA and CACA were recorded on DUM neuron cell bodies. All of these observations are consistent with the possible existence of two GABA-gated chloride channel subtypes in the insect CNS.

Keywords: cockroach; GABA; chloride channel; neuron; picrotoxin; *cis*-4-aminocrotonic acid

1 INTRODUCTION

The inhibitory neurotransmitter 4-aminobutyric acid (GABA) is widely distributed in the insect central nervous system (CNS). Most GABA receptors in insects relate to GABA-gated chloride channels that share some analogies with vertebrate $GABA_A$ receptor subtypes, despite their insensitivity to bicuculline.[1–4] This GABA receptor subtype is found at synapses and on synapse-free cell bodies (extrasynaptic receptors) of neurons. However, in a ventral giant interneuron (GI) of the cockroach nerve cord, two subtypes of GABA-operated chloride channel receptors have been hypothesized on account of differences in chloride conductance increase, GABA and picrotoxin (PTX) sensitivity and distribution on GI dendrites.[5] To characterize further the properties of an insect GABA-gated chloride channel receptor subtype that is weakly sensitive to PTX, electrophysiological and pharmacological experiments were performed on synaptic GABA receptors in an identified ventral GI and on somatic GABA receptors in dorsal unpaired median (DUM) neurons and the fast coxal depressor (D_f) motoneuron of the cockroach, *Periplaneta americana* (L). The native GABA-gated chloride channel receptor subtype resistant to PTX reported here shares some of the pharmacological characteristics, including sensitivity to *cis*-4-aminocrotonic acid (CACA), and some physiological properties of vertebrate $GABA_C$ receptors[6–9] and also of mutated dieldrin-resistant (Rdl) GABA receptor homo-oligomers of *Drosophila*

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melanogaster transiently expressed in *Xenopus* oocytes,[10] or stably expressed in a *D melanogaster* cell line,[11] or cultured neurons from mutant *Drosophila*.[12]

2. MATERIALS AND METHODS

All experiments were performed at room temperature (20–23 °C) using adult male cockroaches (*P americana*) reared at 28 °C on a 12h:12h light:dark cycle.

2.1 Oil-gap recordings

The single-fibre oil-gap method[13] was used to record the GABA responses on synaptic receptors. The terminal abdominal ganglion (TAG), one cercus and the corresponding cercal nerve XI were isolated. The TAG was carefully desheathed to facilitate penetration of micropipettes or bath-applied drugs. The GI2 (according to the nomenclature of Harris and Smyth[14]) was isolated as close as possible to the TAG. The nerve preparation was mounted in a Perspex chamber suitable for the single-fibre oil-gap method. The TAG was continuously superfused at a constant rate (1 ml min^{-1}) with saline of the following composition NaCl (208), KCl (3.1), $CaCl_2$ (5.4), $NaHCO_3$ (2), HEPES (10), sucrose (26 mM), pH 7.4. Direct measurements of membrane conductance changes were achieved using a Wheatstone bridge circuit allowing application of hyperpolarizing square current pulses (5 nA) through the cell membrane. GABA and CACA were ejected within the TAG neuropile using broken micropipettes (tip diameter 5 μm) connected to a pneumatic pressure-ejection system (Neurophore BH2, Medical System Corp, Greenvale, NY) delivering repetitive pulses of nitrogen. Direct micro-applications allow precise local applications of drugs and facilitate the reversibility of the induced effects. The dose of agonist was varied by changing either the duration or pressure of the repetitive gas pulse.[15]

2.2 Intracellular recordings

2.2.1 D_f motoneuron

The metathoracic ganglion was isolated, desheathed in the following saline: NaCl (210), KCl (3.1), $CaCl_2$ (9), sucrose (60), TES (10 mM), pH 7.2 and mounted in the experimental chamber as described previously.[16] One of the paired D_f motoneurons was visually located and impaled with a glass microelectrode filled with 1 M potassium acetate (resistance 15–20 MΩ). Repetitive pulses of agonists (either GABA or CACA) were delivered to the cell by pressure ejection, using a broken micropipette whose tip (diameter: 20–30 μm) was positioned 100–300 μm above the cell body. The control resting potential of the D_f motoneuron was −70 to −85 mV.

2.2.2 DUM neuron

Electrophysiological recordings were performed on in-situ DUM neuron cell bodies in the TAG.[17] The abdominal nerve cord and its desheathed ganglion were mounted in the experimental chamber and superfused with a saline of the following composition. NaCl (200), KCl (3.1), $CaCl_2$ (5), $MgCl_2$ (4), sucrose (50), HEPES (10 mM) pH 7.4. The electrical activity of DUM neurons was recorded through intracellular microelectrodes with resistance 45–65 MΩ when filled with 1 M potassium acetate. Agonists were applied as described in the above paragraph. The normal resting potential of DUM neurons was −50 to −60 mV.

Picrotoxin (PTX) was dissolved in the corresponding saline and externally applied when necessary. All compounds were purchased from Sigma Chemicals except CACA which was obtained from Tocris (Cookson Ltd, Bristol, UK). When quantified, the results were expressed as mean (±SEM). Although many of the figures show results from single experiments, we confirmed each observation in at least three different preparations.

3 RESULTS

3.1 GABA-induced postsynaptic membrane conductance changes

The measure of the membrane conductance changes in response to micropressure application of GABA was firstly carried on GI post-synaptic membranes. Long-lasting (30 s) cyclic pressure microapplication of 10^{-2} M GABA onto GI dendrites reversibly enhanced the post-synaptic membrane conductance to chloride ions as already shown.[5] It appeared that the time-course of the GABA-induced responses was dose-related. High doses evoked a biphasic response, including an early peak followed by a lower steady state (Fig 1A), whereas lower doses of GABA produced a sustained decrease of the membrane resistance (Fig 1B). In order to separate the two GABA responses, a dose-response curve representing the normalized percentage of the membrane conductance change *versus* applied GABA was plotted. In this respect, the peak (4 s after the onset of the GABA response) and steady state (end of the GABA microapplication) were measured in representative experiments ($n = 12$). The points fitted two sigmoidal curves with a common relationship for low doses of GABA (Fig 1). Both phases of GABA-evoked responses might be due to a rapid glial uptake of GABA[18] and/or to the presence of two GABA-gated chloride channel receptor subtypes that differ in their desensitization process. Pharmacological assays described in the following sections will provide conclusive data in support of the latter.

3.2 Pharmacological characterization of GABA-induced response

As previously described in the cockroach TAG[19] bicuculline did not modify the GABA-induced membrane conductance increase, measured at the early peak and during the sustained response, suggesting that cockroach GABA-gated chloride channel receptors are insensitive to bicuculline. To date all the

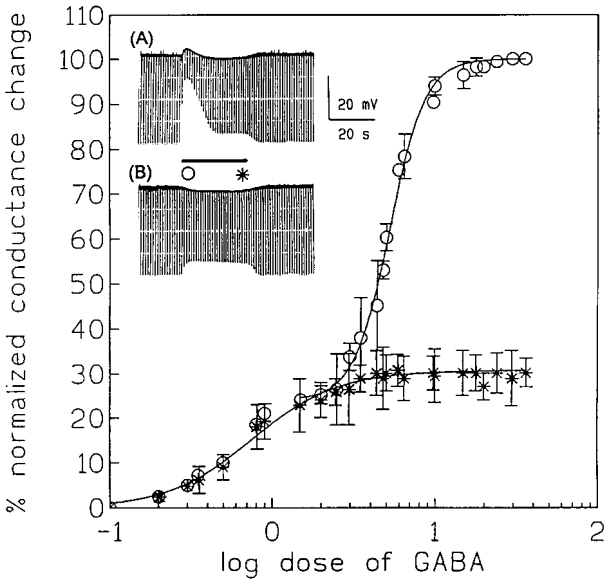

Figure 1. Effects of GABA on GI synaptic GABA-gated chloride channel receptors. Dose-response curves for GABA (in mol litre^{-1}) on GI postsynaptic membrane were plotted against (○) the resultant peak and (*) steady state membrane conductance decreases measured by hyperpolarizing potential (downward deflection) induced by constant square current pulses (5 nA). The mean GABA-mediated membrane conductance changes (±SEM, n=12), were expressed as a percentage of the maximum GABA response. The curves were fitted using non-linear regression analysis. Inset:. A: High dose of GABA induced a biphasic increase in membrane conductance involving an early peak representing the maximum GABA response. B: Low dose of GABA induced a stable increase in membrane conductance reaching 30% of the maximum GABA response. Wide horizontal bar: duration of GABA application.

GABA-gated chloride channel receptors studied were readily blocked by high doses (10^{-4}M) of PTX.[4,5] Lower doses of PTX revealed an insensitivity of certain categories of synaptic GABA-operated chloride channel receptors.[5] This latter point paralleled the differential sensitivity to dieldrin of mutated GABA receptors from *Drosophila*.[10] Furthermore, it has been shown that the LCCH3 subunit, which has high sequence identity to vertebrate beta-$GABA_A$ receptor subunits, was found early in development in the majority of neuroblasts and later was localized as an extrasynaptic receptor on the neuronal cell bodies surrounding the adult brain.[20] In contrast, Rdl receptor subunits appear confined to the neuropile (synaptic area) of the ganglion.[20] Two types of experimental neuronal model were chosen because of this possible difference in molecular composition of the GABA receptor under test.

In synaptic GI preparations, treatment with 10^{-5}M PTX blocked the phasic GABA-induced membrane conductance changes, revealing a residual response (not illustrated) which was sustained. However higher doses of PTX (10^{-4}M) markedly reduced this sustained conductance decrease. The steady-state response and its low sensitivity to PTX was also evoked by CACA, known to preferentially activate the vertebrate $GABA_C$-gated chloride channel receptors subtype[6,8] (Fig 2). Because this membrane conductance increase, accompanied by a steady-state hyperpolarization, was reversed close to the equilibrium potential for chloride ions,[5] it was concluded that chloride ions were involved in the GABA/CACA-induced steady-state membrane conductance increase. It might be suggested that the peak component of the GABA-induced membrane conductance increase is pharmacologically distinct from the sustained response, and to be attributed to two distinct GABA-gated chloride channel receptor subtypes. To assess the possible existence of extrasynaptic PTX-resistant GABA-gated chloride channel receptors, electropharmacological tests were performed on somatic neuronal membranes of D_f and DUM neurons.

3.3 Extrasynaptic GABA receptors

Figure 3A shows a typical response in the cell body of motoneuron D_f to a long-lasting (3 min) cyclic (20 ms on, 40 ms off) pressure microejection of GABA (2×10^{-2}M). The biphasic response comprised an initial fast transient hyperpolarization followed by a sustained hyperpolarization. PTX (10^{-5}M) blocked the fast transient hyperpolarization but had no effect on the slow hyperpolarization. This last response was not quickly desensitized during GABA application. When PTX was bath-applied at 10^{-4}M the slow depolarization was reduced, but a PTX-resistant component was still visible. At the higher concentration PTX caused the neuron to generate spontaneous plateau potentials previously described in the motoneuron Df.[21] Since long-lasting local applications of GABA in absence or presence of PTX mimicked the effect of GABA on the postsynaptic membrane,[5] it is likely that the GABA-induced response in D_f is triggered by different GABA-gated receptors. To further characterize the components of GABA-gated receptors in D_f, CACA was used. Pressure-ejection of CACA (2×10^{-2}M) for 3 min evoked a slow sustained hyperpolarization that was not quickly desensitized (Fig 3B). The CACA-induced response was partially inhibited with PTX (10^{-6}M) and was fully blocked by PTX (10^{-5}M) (Fig 3B). Because CACA was unable to induce the fast transient hyperpolarization but a slow hyperpolarizing response resembling the GABA-in-

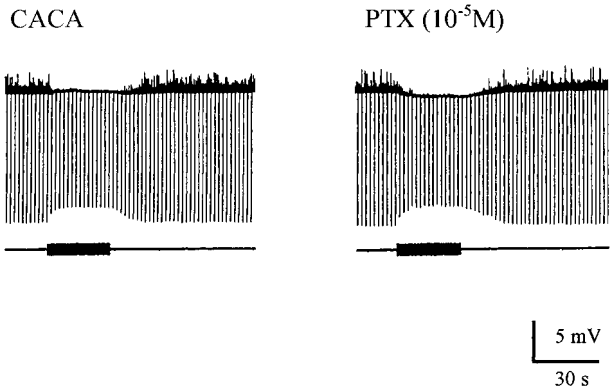

Figure 2. *cis*-4-Aminocrotonic acid (CACA) induces PTX (10^{-5}M) -resistant sustained membrane conductance increase in cockroach GI.

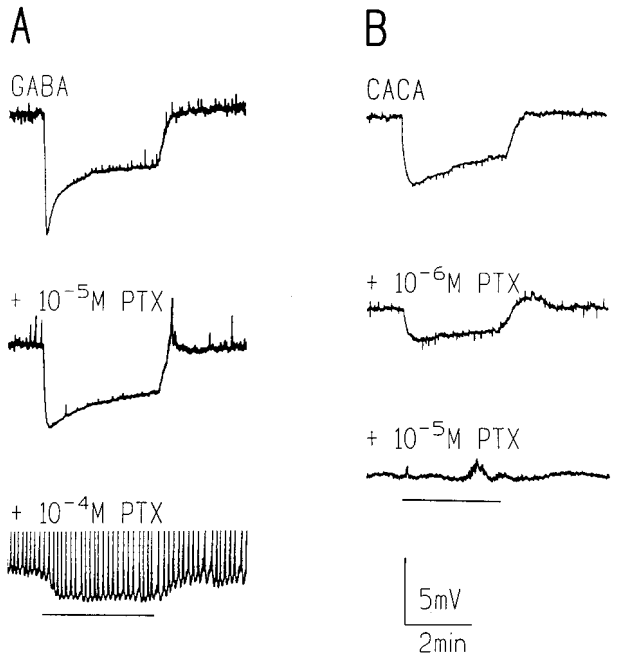

Figure 3. Effects of GABA and *cis*-4-aminocrotonic acid (CACA) in motoneuron D_f. (A) Pressure ejection of GABA (2×10^{-2}M) or (B) CACA (2×10^{-2}M) for 3min (lower horizontal bar), alone or in presence of PTX. The results were recorded from two different preparations.

duced response in PTX (10^{-5}M), it was concluded that the fast transient component was pharmacologically distinct from the sustained response. The PTX sensitivity of both components supported chloride currents.

GABA and CACA were also tested on DUM neuron cell bodies to verify if different GABA responses were present on a different neuronal type. As described for motoneurone D_f, pressure ejection GABA (2×10^{-2}M) (Fig 4A) or CACA (2×10^{-2}M) (Fig 4B) evoked a fast transient hyperpolarization of DUM neuronal cell bodies followed by a sustained phase or a slow sustained hyperpolarization respectively. Although PTX (10^{-5}M) antagonized the fast transient response, unmasking the slow hyperpolarization characterized by a limited desensitization during GABA ejection, higher concentrations of PTX (10^{-4}M) had little inhibitory effect on the second phase. Despite insensitivity of the slow component to PTX, it seems that a sustained chloride current may be present in DUM neurons since CACA evoked a sustained hyperpolarization fully blocked by PTX at 10^{-5}M (Fig 4B).

4. DISCUSSION

It is well established that GABA receptors form important targets for drugs and toxicants in both vertebrates and invertebrates. However, insect GABA receptors present pharmacological differences from vertebrate receptors[1,2] which might allow for the development of specific insecticide molecules. The above results describe, on three different cockroach neuronal preparations, specific characteristics of a native PTX-resistant GABA-gated chloride channel receptor subtype which extends previously reported evidence for the presence of two GABA receptor subtypes in GI postsynaptic membrane.[5] Several experimental arguments support this conclusion. Firstly it has been demonstrated that the GABA-induced membrane response is composed of a fast transient hyperpolarization followed by a submaximal response that is not quickly desensitized during long-lasting GABA application. Furthermore, PTX (10^{-5}M), which antagonized the fast transient GABA-induced response, did not affect the sustained response which reached a low maximal membrane conductance increase. Finally, the specific vertebrate $GABA_C$ agonist CACA induced the PTX-resistant sustained response in all neuronal preparations tested.

Although this PTX-resistant GABA-operated chloride channel receptor subtype shares some of the physiological and pharmacological properties of the vertebrate $GABA_C$ receptor and of *Drosophila* Rdl mutated GABA receptor, its physiological role is not clear. Nevertheless, it is of interest to consider that this GABA receptor subtype, identified in the cockroach

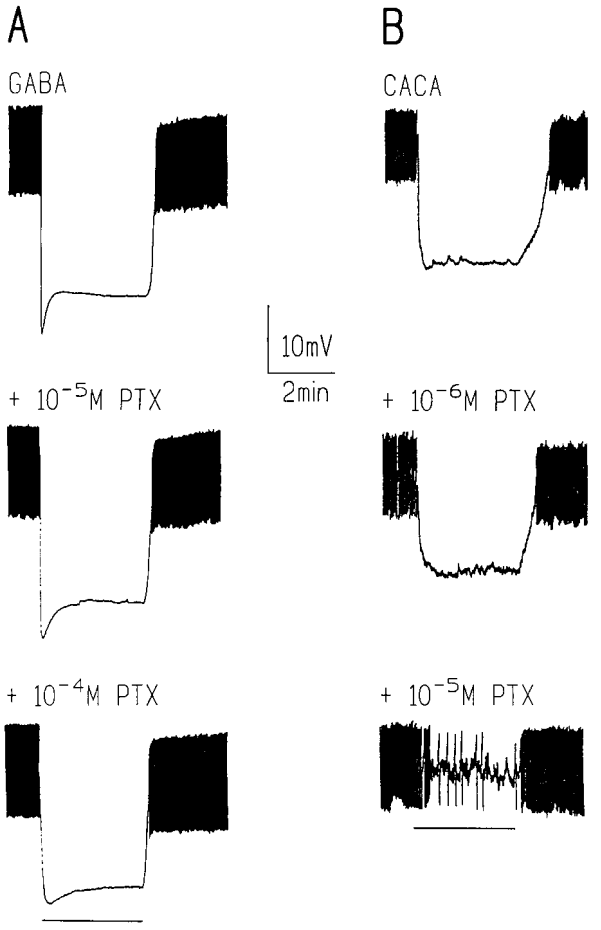

Figure 4. Hyperpolarizing effects of GABA and *cis*-4-aminocrotonic acid (CACA) in DUM neurons. Pressure ejection of (A) GABA (2×10^{-2}M) or (B) CACA (2×10^{-2}M) for 3min (lower horizontal bar), alone or in presence of PTX. The thick line represents action potentials which were attenuated because the chart recorder had slow characteristics. The results were recorded from two different preparations.

CNS, may represent a specific target site for new insecticides.

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